

The *Drosophila* γ -Tubulin Small Complex Subunit Dgrip84 Is Required for Structural and Functional Integrity of the Spindle Apparatus[□]

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γ -Tubulin, a protein critical for microtubule assembly, functions within multiprotein complexes. However, little is known about the respective role of γ -tubulin partners in metazoans. For the first time in a multicellular organism, we have investigated the function of Dgrip84, the *Drosophila* orthologue of the *Saccharomyces cerevisiae* γ -tubulin-associated protein Spc97p. Mutant analysis shows that Dgrip84 is essential for viability. Its depletion promotes a moderate increase in the mitotic index, correlated with the appearance of monopolar or unpolarized spindles, impairment of centrosome maturation, and increase of polyploid nuclei. This *in vivo* study is strengthened by an RNA interference approach in cultured S2 cells. Electron microscopy analysis suggests that monopolar spindles might result from a failure of centrosome separation and an unusual microtubule assembly pathway via centriolar triplets. Moreover, we point to an involvement of Dgrip84 in the spindle checkpoint regulation and in the maintenance of interphase microtubule dynamics. Dgrip84 also seems essential for male meiosis, ensuring spindle bipolarity and correct completion of cytokinesis. These data sustain that Dgrip84 is required in some aspects of microtubule dynamics and organization both in interphase and mitosis. The nature of a minimal γ -tubulin complex necessary for proper microtubule organization in the metazoans is discussed.

INTRODUCTION

The mechanisms of microtubule nucleation remain unclear, although it has been demonstrated that γ -tubulin, a universal component of the microtubule-organizing centers, plays an essential role in microtubule nucleation. The molecular details of this process are still poorly understood (Oakley and Oakley, 1989; Oakley *et al.*, 1990; Erickson and Stoffler, 1996; Gunawardane *et al.*, 2003). *In vitro*, γ -tubulin monomers enhance the assembly of α/β heterodimers and block the minus ends of microtubules (Li and Joshi, 1995; Leguy *et al.*, 2000). However, *in vivo*, γ -tubulin does not seem to act as a monomer, but rather in a variety of protein complexes (Murphy *et al.*, 1998; Oegema *et al.*, 1999; Fujita *et al.*, 2002). The simplest one called γ -tubulin small complex (γ -TuSC) has been well characterized in *Saccharomyces cerevisiae* (Knop

et al., 1997), *Drosophila melanogaster* (Oegema *et al.*, 1999), and vertebrates (Murphy *et al.*, 1998). It is formed by γ -tubulin and two associated proteins in a 2:1:1 stoichiometry (Knop and Schiebel, 1997; Oegema *et al.*, 1999; Murphy *et al.*, 2001). This small complex is recruited at the inner and outer spindle plaques of *S. cerevisiae* spindle pole bodies (SPB) where it is responsible for microtubule nucleation (Knop and Schiebel, 1997, 1998; Pereira *et al.*, 1998). Besides γ -TuSC, other larger γ -tubulin-containing complexes have been described (Zheng *et al.*, 1995; Fujita *et al.*, 2002). One termed γ -tubulin ring complex (γ -TuRC) has been characterized in multicellular organisms such as *D. melanogaster*, *Xenopus laevis*, and *Homo sapiens* (Zheng *et al.*, 1995; Oegema *et al.*, 1999; Murphy *et al.*, 2001). It is assumed that the γ -TuRC results from the association of several γ -TuSCs with at least four other proteins. Three of them show sequence homologies (grip motifs) with the two γ -tubulin-associated proteins in the γ -TuSC, whereas the fourth exhibits five WD repeats (Fava *et al.*, 1999; Gunawardane *et al.*, 2000; Moritz *et al.*, 2000; Murphy *et al.*, 2001; Gunawardane *et al.*, 2003). The function of γ -tubulin has been studied extensively in several organisms and in cultured cells using antibody microinjection, mutants or gene silencing (Oakley *et al.*, 1990; Horio *et al.*, 1991; Joshi *et al.*, 1992; Sunkel *et al.*, 1995; Marschall *et al.*, 1996; Spang *et al.*, 1996; Wilson and Borisy, 1998; Llamazares

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et al., 1999; Ruiz *et al.*, 1999; Bobinnec *et al.*, 2000; Sampaio *et al.*, 2001; Raynaud-Messina *et al.*, 2004). For example, in *Drosophila* where two γ -tubulin isotypes are expressed, homozygous γ -tub 23C mutants die during late larval stage, exhibiting atypical mitotic spindles and abnormal centrosomal structures (Sunkel *et al.*, 1995), whereas disruption of the γ -tub 37CD gene results in abnormal female meiotic spindles (Tavosanis *et al.*, 1997). However, our knowledge about the function of the γ -tubulin-interacting proteins is limited. The role of the two grip proteins associated with γ -tubulin in the γ -TuSC has been studied both in budding and fission yeasts (Geissler *et al.*, 1996; Knop *et al.*, 1997; Nguyen *et al.*, 1998; Vardy and Toda, 2000; Vardy *et al.*, 2002). Deletions of each of these genes (SPC97 and SPC98 in *S. cerevisiae*, *Alp4* and *Alp6* in *Schizosaccharomyces pombe*) are lethal; they induce mitotic defects and abnormal long cytoplasmic microtubules. However conditional mutants show phenotypic differences (Geissler *et al.*, 1996; Marschall *et al.*, 1996; Spang *et al.*, 1996; Knop *et al.*, 1997). In *S. cerevisiae*, SPC98p seems critical for γ -tubulin anchorage to the inner spindle plaque via direct interaction with SPC110p (Nguyen *et al.*, 1998), whereas SPC97p seems essential for a correct SPB duplication and separation (Knop *et al.*, 1997). In fission yeast, *Alp4* mutations compromise γ -tubulin localization to the SPB, but they do not affect the assembly of the large γ -tubulin complex (Vardy and Toda, 2000). These studies reveal discrepancies about the role of the two grip motif γ -TuSC subunits, in particular in the SPB duplication/separation process and in the γ -tubulin anchorage. Moreover, these results obtained in yeasts are difficult to transfer to metazoans for several reasons. First, the morphology of the microtubule-organizing centers is different. Second, the amino acid sequences of these proteins are poorly conserved because SPC97p and its *Drosophila* orthologue (Dgrip84) exhibit only 10% identity and 22% similarity. Third, in contrast with *S. cerevisiae* where γ -tubulin relocates to the SPB as γ -TuSC, in multicellular organisms, it is assumed that γ -tubulin is recruited to the centrosome as γ -TuRC. In metazoans, the silencing of γ -tubulin-associated proteins (Dgrip91, the SPC98p orthologue, and Dgrip75) has been performed essentially in *Drosophila* and does not induce similar phenotypes (Barbosa *et al.*, 2000; Schnorrer *et al.*, 2002). Dgrip91 is an essential protein, required for correct bipolar spindle assembly during mitosis and male meiosis (Barbosa *et al.*, 2000, 2003). In contrast, Dgrip75, a protein restricted to the large γ -tubulin complex, is essential for female fertility, but not for viability. Mutations in *Dgrip75* prevent the correct localization of some morphogenetic determinants during oogenesis, suggesting a role in the organization/dynamics of some subsets of microtubules (Schnorrer *et al.*, 2002).

In this study, we have performed a functional analysis of Dgrip84 during mitosis and male meiosis using two independent strategies (mutant strains and RNA interference [RNAi] in cultured cells). We have also approached the role of this γ -TuSC protein in the organization of microtubule cytoplasmic arrays. Dgrip84, as γ -tubulin and Dgrip91, is essential for viability attesting that none of these three proteins is fully redundant.

MATERIALS AND METHODS

Cell Culture and RNA-mediated Interference

RNA interference treatments were performed in S2 cells (Schneider, 1972) according to Clemens *et al.* (2000). Cells were treated twice with RNAi against *Dgrip84* at days 1 and 5, and harvested on day 7 for immunoblotting and immunofluorescence staining. *Dgrip84* double-strand RNA (dsRNA) corresponding to positions 1–756 relative to the start of translation was used.

Comparable results have been obtained with dsRNA corresponding to positions 885–1580 (our unpublished data). These dsRNA were generated from the cDNA clone LD12257 as described in Raynaud-Messina *et al.* (2004). For cold microtubule depolymerization, cells were maintained for 2 h in melting ice. For drug depolymerization, colchicine (Sigma-Aldrich, St. Louis, MO) was added to culture medium to a final concentration of 25 μ M and then incubated for 16 h.

Fly Strains and Generation of the R20 Deletion

Strains y^1w^{118} and w^{1118} were used as controls, whereas strains *l(1)PG36* (PG36/FM7) and *l(1)PG66* (PG36/FM7), carrying P-element insertions (Bourbon *et al.*, 2002), were named PG36 and PG66, respectively. To recover excision alleles, the PG66 element was mobilized by generating dysgenic females carrying the $P[ry^+, \Delta 2-3]99B$ *Sb* chromosome (Robertson *et al.*, 1988), crossing them with w^- FM7 males, and recovering w^- *Sb*⁺ FM7 balancer females. Male lethal strains were kept. One among these new *Dgrip84* mutant lines, named R20 (R20/FM7), carries a genomic deletion that has been characterized by PCR on single whole third instar (L3) extracts (Gloor *et al.*, 1993). For this purpose, we used gene-specific primers complementary to sequences (available upon request) localized between 1 kb 5' of the translation start codon and the translation stop codon. Moreover, each of the mutant chromosomes, PG36, PG66, and R20 has been balanced over FM7, $P[Kr:Gal4]$, $P[UAS:GFP]$ (Casso *et al.*, 2000). These strains were used to select the male mutant larvae, which were not fluorescent under the stereomicroscope because they did not possess the green fluorescent protein (GFP)-carrying balancer chromosome.

P-Element-mediated Germline Transformation and Tests for Rescuing Dgrip84 Lethality

The cDNA clone RE10613 was sequenced using internal primers designed specifically for that purpose. Compared with the genomic DNA sequenced by the Berkeley *Drosophila* Genome Project, two point mutations were detected. Thus, the 4.7-kb *NsiI/BglIII* fragment from RE10613 was subcloned into the LD12257 incomplete clone (digested by *NsiI/BglIII*) to get a wild-type complete clone, termed RE84. A 2.7-kb *EcoRI/XbaI* fragment from the RE84 clone was subcloned into the pUbbH1 vector (Calgario *et al.*, 2002) to yield the pUbb84 construct. The 5.2-kb *NotI/XmnI* fragment from pUbb84 was subcloned into the *NotI/StuI* site of the transformation vector pCaSpeR (Thummel and Pirrotta, 1992), to give rise to the final rescue construct, $P[Ubb84,w^+]$. W^- embryos were coinjected with $P[Ubb84,w^+]$ and pUChs $\Delta 2-3$ (Mullins *et al.*, 1989), by standard techniques (Rubin and Spradling, 1982). Emerging adults were crossed individually to w^- flies, and w^+ transformant progeny was selected. More than 20 independent lines were recovered, chromosomal linkage was determined by crosses with a multiple balancer stock, and homozygous stocks were established. Three transformant lines carrying the $P[Ubb84,w^+]$ construct on the second chromosome (lines 10, 12, and 17) were tested for rescuing the lethality of PG36, PG66, and R20 hemizygous males. Rescue was assessed by scoring for surviving male progeny with the genotype w^+ , *Dgrip84*⁻/^Y; $P[Ubb84,w^+]$ /+.

Antibodies

The mouse monoclonal antibodies T-5168 (Sigma-Aldrich) and 1501 (Chemicon International, Temecula, CA) were used to stain for α -tubulin and actin, respectively. The following rabbit polyclonal antibodies were used: Rb3133 against Asp (Saunders *et al.*, 1997) and GM2 against Pav-KLP (Minestrini *et al.*, 2003; gifts from Dr. Glover, University of Cambridge, Cambridge, United Kingdom), R19 against Cnn (Heuer *et al.*, 1995; gift from Dr. Kaufman, Howard Hughes Medical Institute, Indiana University, Bloomington, IN), Cid against a H3-like protein used as a centromere identifier (Henikoff *et al.*, 2000; gift from Dr. Henikoff, Howard Hughes Medical Institute, Seattle, WA), Rb666 against BubR1 (Logarinho *et al.*, 2004), R62 against 23C γ -tubulin (Raynaud-Messina *et al.*, 2001), R522 against Dgrip84, and R7075 against Dgrip91. R522 was raised against the 15 carboxy-terminal amino acids of Dgrip84 and affinity purified on recombinant Dgrip84, produced in *Escherichia coli* from the clone pRE84. Under the conditions used for Western blotting of cultured S2 cells and L3 larval brain extracts, R522 antibodies specifically recognized a polypeptide with an apparent mass of 97 kDa. This labeling was abolished when antibodies were preincubated with the antigenic peptide. R7075 was raised against the 414–917 amino acid region of Dgrip91.

Western Blotting

Protein extracts from cultured S2 cells (Raynaud-Messina *et al.*, 2001) and from total brains were subjected to Western blot analyses as described previously. Total brain extracts were prepared from about 10 brains of each genotype, dissected in saline (0.7% NaCl), boiled for 3 min in 2 \times Laemmli sample buffer and diluted twice.

Cytological Analyses

The percentages of the different mitotic phenotypes were determined with confidence intervals calculated for a probability of 95%. For immunostaining,

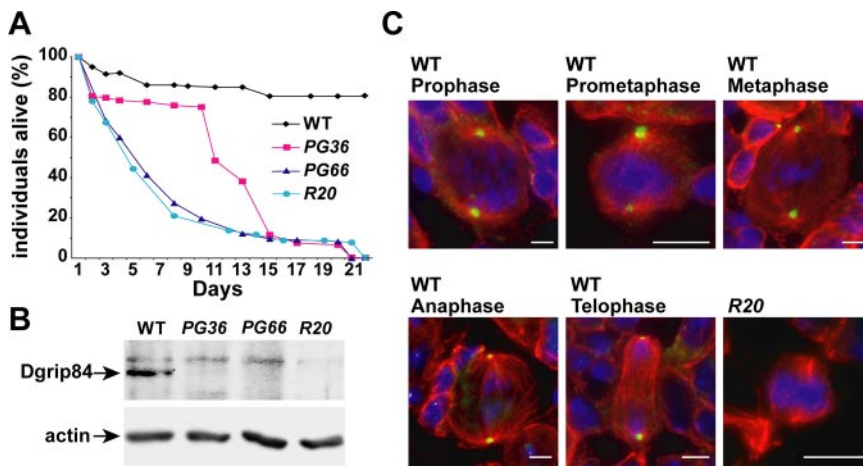


Figure 1. Characterization of *Dgrip84* mutants. (A) The three *Dgrip84* mutations induce different patterns of lethality. One hundred first instars of each mutant genotype and wild-type (WT) were followed until adult stage. The percentage of live individuals—independently of the developmental stage—was determined. The *PG66* and *R20* alleles induce an early lethality, whereas the *PG36* allele induces a belated lethality. (B) *Dgrip84* protein is not detectable in the brain of the three mutants. Total protein extracts from L3 larval brains (~10 brains for each genotype) were submitted to Western blot analyses with affinity-purified *Dgrip84* antibodies (top). Actin was used as an internal loading control (bottom). (C) *Dgrip84* protein is recruited at the centrosome during mitosis in wild-type larval brains but was not detectable in larval *Dgrip84* mutant neuroblasts. L3 larval brains were probed with affinity-purified antibodies

against *Dgrip84* protein (green). Wild-type neuroblasts showed *Dgrip84* protein recruitment at the centrosome throughout mitosis, whereas *Dgrip84* is not detectable at the poles in *R20* mutant neuroblasts at any mitotic stage. In all figures except Figure 6, the spindle is shown in red (anti- α -tubulin antibodies) and chromosomes in blue (DAPI staining). Bars, 5 μ m.

cultured S2 cells were fixed in DES culture medium (Invitrogen, Cergy Pontoise, France) containing 3.7% vol/vol paraformaldehyde (20 min for antibodies R62, R522, Rb666, and Rb3133; 2 min for antibodies Cid, R7075, and R19), permeabilized for 2 min in methanol (–20°C) (Raynaud-Messina *et al.*, 2004), and then incubated with antibodies. For Cid staining, 1-min preincubation in DES medium containing 0.05% Triton X-100 and 0.1 mM CaCl_2 was performed. Mitotic indices were quantified after 4,6-diamidino-2-phenylindole (DAPI) staining and α -tubulin (or Centrosomin) immunofluorescence analysis on fixed preparations or by mitotic spread of the whole cellular suspension stained with DAPI. Immunostaining of semisquashed L3 larval brains was performed as described in Bonaccorsi *et al.* (2000). DAPI staining of squashed L3 larval brains were made as described previously (Sunkel *et al.*, 1995). Testes immunostaining was carried out as described in Pisano *et al.* (1993). For analysis of live testes by phase contrast microscopy, testes were dissected in 0.7% NaCl, transferred onto a clean slide in a drop of NaCl, and cut with tungsten needles near the apical tip to release the cysts through the testes wall. The testes were gently squashed on the slide with a coverslip by applying a small piece of blotting paper to one of the edges of the coverslip, while monitoring under a 40 \times phase contrast objective. Testes were obtained from young dark pupae (*PG36*), young adults (*PG36*), or L3 larvae (*PG66* and *R20*). For electron microscopy, cells were fixed and treated as described (Raynaud-Messina *et al.*, 2004).

RESULTS

Characterization of *Dgrip84* mutants

A screen carried out to generate recessive lethal *P*-element insertions within X-linked genes allowed the isolation of two *Dgrip84* mutant alleles, *PG66* and *PG36* (Bourbon *et al.*, 2002). These two insertions are located at positions –240 and –350 base pairs, respectively, from the translation initiation ATG codon of the previously described *Dgrip84* transcript (Oegema *et al.*, 1999). To ensure that the lethality is due to the insertion event, the *PG66* element was remobilized, allowing the isolation of viable *w⁻* revertants, from which the *w⁺* transposon has been precisely excised. Among the generated imprecise excisions of the *P*-element, we recovered a new lethal allele, *R20*, which bears a deletion covering a large section of the coding sequence, including the initiation ATG. Finally, three independent transgenic strains, expressing the wild-type *Dgrip84* protein under the control of the *ubiquitin-63E* promoter (see *Materials and Methods*), are able to complement the lethality induced by all three mutant alleles, demonstrating that *Dgrip84* is essential for viability.

Analysis of lethal phases indicates that *R20* and *PG66* mutants exhibit early lethality (Figure 1A), dying mainly during the first and second instars. In contrast, *PG36* seems

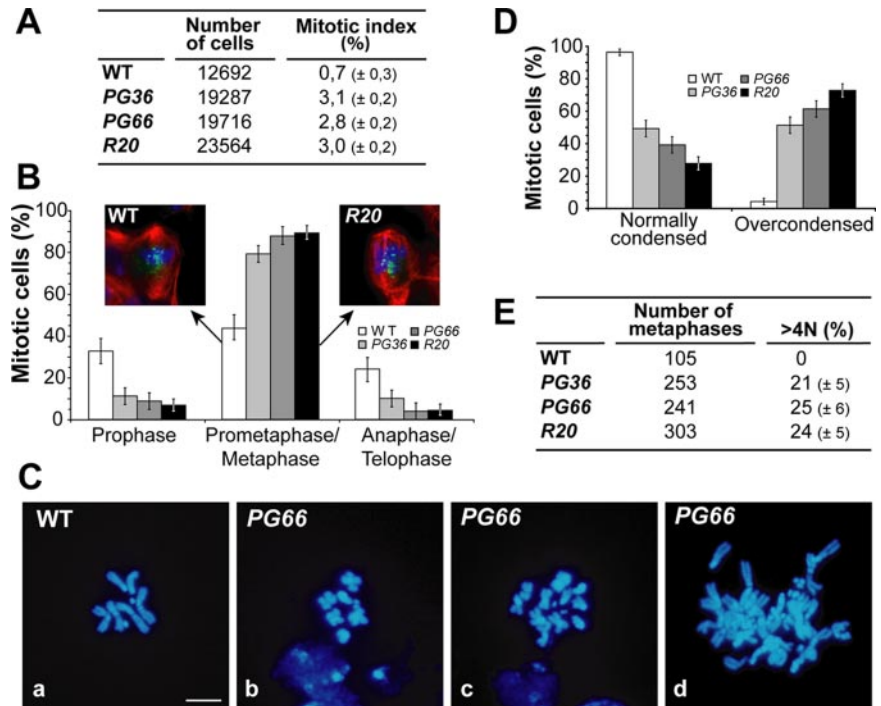
to be semilethal (Figure 1A), allowing 50% of hemizygous males to reach the adult stage after a 2-d delay. These mutant males show reduced viability (Figure 1A), are sterile, and exhibit abnormalities in the abdominal cuticle and the thoracic macrochaete pattern (our unpublished data), which are common in mutations affecting mitosis. Together, these observations suggest an allelic series with the progression $PG36 < PG66 \leq R20$.

Dgrip84 protein level was determined for the different alleles. Total protein extracts from wild-type L3 larval brains as well as from hemizygous *Dgrip84* mutant brains were probed with *Dgrip84* antibodies (Figure 1B, top). Compared with the signal obtained in wild-type brain extracts, *Dgrip84* level is strongly reduced below the threshold of detection in the three mutants. Immunofluorescence analysis shows that in wild-type L3 larval brains *Dgrip84* protein is undetectable during interphase (our unpublished data) and is present at the spindle poles throughout mitosis (Figure 1C). In contrast, 99% of *R20* mitotic cells are devoid of *Dgrip84* signal ($n = 468$) (Figure 1C) consistently with the decrease of *Dgrip84* levels on Western blots. Similar data were observed with *PG36* (our unpublished data). These results indicate that *Dgrip84* alleles are either null (*R20*) or severe hypomorphic alleles (*PG36* and *PG66*).

Dgrip84 Mutations Delay Progression through Mitosis

Because γ -tubulin complexes are necessary for cell division, mitotic phenotypes have been analyzed in L3 larval brains. For the three *Dgrip84* mutants, the mitotic index is elevated four- to fivefold (Figure 2A), independently of the progression of the allelic series. Cells accumulate in prometaphases/metaphases, whereas the percentage of anaphases/telophases decreases (Figure 2B). In all prometaphase/metaphase mutant cells (98–100%, $p = 95\%$), the mitotic checkpoint is activated as evidenced by staining of kinetochores with BubR1 antibodies (Figure 2B, inset). More than half of the mutant cells exhibit overcondensed chromosomes (Figure 2C, b and c, and D), consistently with a delay in the progression through prometaphase/metaphase stages. Most of the mutant cells show disorganized metaphase plates (Figure 2C, c and d). The occurrence of ~25% of cells with DNA content superior to 4N (Figure 2C, c and d, and E)

Figure 2. Mitotic phenotypes in *Dgrip84* mutants. Observations were made on L3 larval neuroblasts. (A) The mitotic index is increased in the three mutants. The percentage of mitotic figures was recorded on five brains for each genotype. Confidence intervals (in parentheses) are calculated for a probability of 95%. (B) Mutant neuroblasts accumulate in prometaphases/metaphases. For each genotype, the different mitotic stages were recorded in >250 cells undergoing mitosis (graph). The inset shows two typical control and mutant prometaphase figures, exhibiting BubR1 (in green) localized at the kinetochores. (C) Abnormal chromosomal plates are observed in R20 mutant neuroblasts. L3 larval brains were squashed and stained with DAPI. a, Wild-type metaphase plate showing eight (4N) normally condensed chromosomes; b, mutant cell showing eight overcondensed chromosomes; c, aneuploid mutant cell with overcondensed chromosomes; and d, hyperploid mutant cell showing ~32 chromosomes (16N). (D and E) Percentage of overcondensed chromosomal figures (D) and the percentage of hyperploid metaphase cells (E) are increased in the three mutants. Five brains were scored for each genotype. Chromosome overcondensation was analyzed in >300 mitotic cells. Bars, 5 μ m.



suggests that these cells escaped the mitotic checkpoint without proper cytokinesis and progressed into a new cell cycle. The number of prometaphases/metaphases and the number of figures with overcondensed chromosomes are significantly lower ($p = 95\%$) in PG36 (the weakest allele) than in R20 (the strongest allele). Overall, these results suggest that Dgrip84 is required for cells to complete successfully mitosis.

Dgrip84 Mutant Cells Display Abnormal Microtubule Organization in Mitosis

Prometaphase delays observed in the *Dgrip84* mutants could be a consequence of defects in the mitotic apparatus. Therefore, we have studied the organization of the spindles in R20 brains using microtubule staining. In comparison with wild-type brains, we observed a large frequency of cells (42%) with a monopolar or a highly abnormal microtubule organization devoid of distinct polarity (Figure 3A; $n = 1622$). Conversely, there is a severe decrease in the frequency of cells with normal bipolar spindles (Figure 3A; $n = 1622$). A large percentage (20%) of these bipolar spindles are asymmetrical as judged by microtubule density or/and polar focalization. Mutant cells show abnormal distribution of Abnormal spindle protein (Asp), a protein that accumulates at the minus ends of microtubules (do Carmo Avides and Glover, 1999; Wakefield *et al.*, 2001; Riparbelli *et al.*, 2002). In wild-type prometaphases, Asp occurs as a regular staining on the side of the centrosome facing the spindle (Figure 3B, a). In R20 mutant brains, most cells with bipolar spindles (88%) show faint Asp signals at the poles (Figure 3B, b). Most monopolar spindles (60%) exhibit a single Asp dot at the pole (Figure 3B, c), whereas the remaining shows abnormal Asp labeling at secondary sites. In cells devoid of microtubule organization, Asp is detected but with an abnormal distribution (Figure 3B, d). In all cases, the signals seem weaker than in wild-type cells. These observations suggest that Dgrip84 is necessary for proper organization of spindle microtubules.

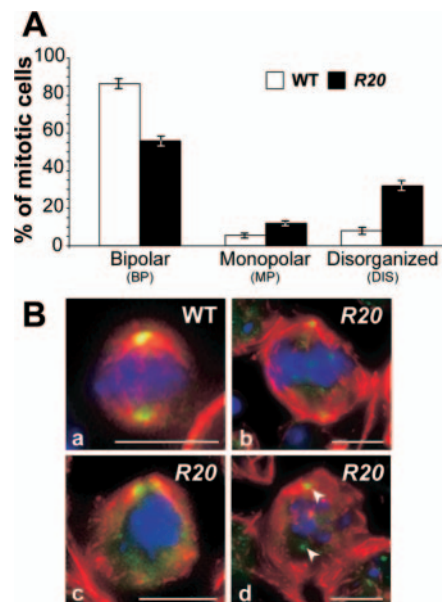


Figure 3. Abnormal mitotic organization in R20 neuroblasts. (A) Percentage of bipolar spindles is decreased, whereas the percentage of monopolar and disorganized figures is increased. The percentage of bipolar (BP), monopolar (MP), and disorganized (DIS) mitotic figures was calculated using spindle labeling (α -tubulin antibodies) of nine L3 larval brains for each genotype (WT, $n = 910$; and R20, $n = 1622$). (B) Mitotic cells exhibit Asp labeling at least at one pole. The Asp signal (green) is weaker in the mutant (b–d) than in the wild type (a). Most bipolar (b; 97%), monopolar (c; 100%), and disorganized mitotic apparatus (d; 93%) exhibit a labeling with Asp antibodies (green). Disorganized microtubule figures exhibited two opposite dots (62%; d, arrowheads), one dot (20%), or several dots (12%). Bars, 5 μ m.

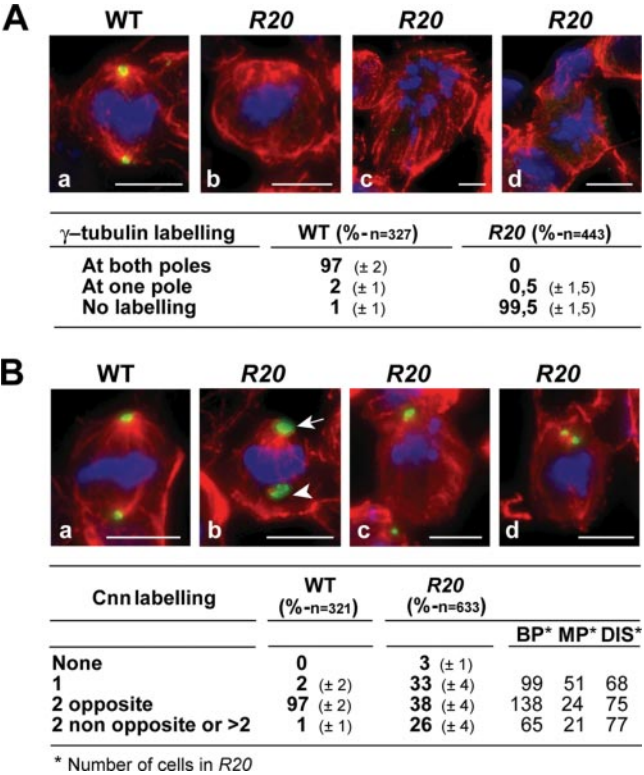


Figure 4. Abnormal centrosome organization in R20 mutants. (A) γ -Tubulin is absent at the poles in mutant mitotic cells. Wild-type mitotic figures (a) show a γ -tubulin signal (green) at both poles. In contrast, in mutant brains, most mitotic cells are devoid of γ -tubulin signal, forming either bipolar spindles (BP; b), monopolar spindles (MP; c), or disorganized mitotic apparatus (DIS; d). A quantitative view is underlying in the table. (B) Localization of Cnn is abnormal in *Dgrip84* mutants. In wild type, the Cnn signal is detected as a single dot at both poles in nearly all mitotic figures (a; table). In mutant brains, the presence of two opposite Cnn dots (b) is observed only in a fraction of mitotic figures (table), whereas mitotic cells often exhibit one dot (c), two nonopposite dots (d), or more than two dots. Besides these abnormalities, Cnn dots can be very large (b; arrow) or can exhibit an abnormal nonspherical shape (b; arrowhead). Bars, 5 μ m.

Dgrip84 Mutant Cells Exhibit Abnormal Centrosome Maturation

γ -Tubulin recruitment at the centrosome was analyzed in R20 mitotic neuroblasts. Whereas γ -tubulin is located at both poles of wild-type spindles, it is no longer detected at the poles in mutant mitotic cells independently of their microtubule organization (Figure 4A). Defects at the spindle poles were confirmed by centrosomin (Cnn) staining (Figure 4B). This centrosomal component, dispersed during interphase, is recruited to the pericentriolar matrix during mitosis (Li and Kaufman, 1996; Megraw *et al.*, 1999). In wild-type neuroblasts, spindle poles labeled with Cnn exhibit a spherical signal (Figure 4B, a) corresponding to functional centrosomes. In R20 mutant brains, one or two Cnn-containing bodies are detected in most mitotic cells (Figure 4B, b–d), in contrast to the complete absence of γ -tubulin in these cells. Cnn-containing bodies show abnormal number, shape, or localization. Some cells contain only one dot, whereas others exhibit two dots at the same pole (Figure 4B, c and d), suggesting that abnormalities in the duplication or/and in the separation of centrosomes might have occurred. Among

Table 1. Quantification of mitotic indices after depletion of Dgrip84 and/or colchicine treatment

	% Cnn labeling	
Control	2.5	(2–3)
RNAi-Dgrip84 treated	6	(5–7)
Control + colchicine	24	(20–28)
RNAi-Dgrip84 treated + colchicine	10	(9–11)

Cells are treated twice with RNAi against Dgrip84, and 16 h before harvest they were exposed to 25 μ M colchicine. Mitotic indices are determined after immunolabeling of the poles with Cnn whose recruitment is insensitive to RNAi and colchicine treatments. Data are representative of two independent experiments. n > 100.

these Cnn-containing bodies, a few have lost the spherical shape and look large (Figure 4B, b, arrow) and/or irregular (Figure 4B, b, arrowhead). Cells with more than two dots are also observed. Some of them exhibit a major fragmented signal or three to eight small Cnn bodies of various sizes dispersed within the mitotic apparatus, suggesting a fragmentation of the pericentriolar material (our unpublished data). Hence, Dgrip84 is likely to be an essential element for the correct maturation and organization of the pericentriolar material during mitosis.

Mitotic Microtubule Organization after Depletion of Dgrip84 in Cultured S2 Cells

In *Dgrip84* mutant strains, even when the maturation of the pericentriolar material is compromised, mitotic spindles, albeit strongly abnormal, are present. To determine how these spindles are generated, we carried out RNA-mediated interference in cultured *Drosophila* S2 cells. Western blot analysis revealed that Dgrip84 is undetectable after a 7-d treatment, whereas the level of actin is not altered.

The mitotic index is increased (4% [3.4–4.6], n = 3771 compared with 1.2% [1–1.4], n = 10,020 in untreated cells). This mitotic arrest coincides with the maintenance of an active mitotic checkpoint as judged by strong BubR1 signals at kinetochores, whatever the spindle morphology (Supplemental Figure A). Although both Dgrip84 mutation and RNAi treatment induce a significant mitotic accumulation, this increase is surprisingly moderate (~3-fold). Three hypotheses could explain this slight blockage: microtubule defects after down-regulation of γ -TuSC proteins are incompetent to trigger an efficient spindle checkpoint; or they promote another cell cycle checkpoint, preventing cells from reaching mitosis; or the γ -TuSC is involved in mitotic checkpoint regulation in a manner independent of microtubule nucleation and attachment functions. To discriminate among these possibilities, we have determined mitotic indices in Dgrip84-depleted cells exposed to a microtubule poison such as colchicine. We scored the mitotic index using three independent parameters: by monitoring chromosome condensation on the whole cell population or on coverslip-fixed cells (data not shown) or by quantifying polar Cnn recruitment (Table 1). Both criteria gave comparable results. Treatment with the anti-microtubule agent blocks cells in mitosis in an efficient manner (~10-fold increase of the mitotic index). Interestingly, this arrest is weakened when cells have been previously submitted to Dgrip84 RNAi (only 4-fold increase compared with control). Together with the increase in aneuploidy in Dgrip84 mutants (see above), these data strongly suggest that Dgrip84 down-regulation induces

Table 2. Quantification of mitotic progression after depletion of Dgrip84

Mitotic stages	Control %	Treated %
Prophases	15 (8–22)	7 (2–12)
Prometaphases		
No polarity	1 (0–3)	10 (5–15)
Monopolar spindles	1 (0–3)	24(16–32)
Bipolar spindles	53(43–63)	54(45–63)
Late stages	30(21–39)	5 (1–9)

Mitotic stages were determined after chromosome staining (DAPI) and immunolabeling of microtubules (α -tubulin antibodies). Data are representative of three independent experiments. $n > 100$.

an untimely exit of mitosis. It could explain, at least partially, why RNAi-treated cells fail to arrest efficiently their mitotic progression. Accordingly with the analyses done in *S. pombe* and *Aspergillus nidulans* (Vardy and Toda, 2000; Hendrickson *et al.*, 2001; Prigozhina *et al.*, 2004), we support the hypothesis of a role of Dgrip84 in spindle checkpoint regulation.

Dgrip84 RNAi treatment affected the overall mitotic organization (Table 2). A main characteristic is a great increase in the percentage of monopolar spindles (Table 2 and Figure 5B, c, f, and i). Although the relative number of bipolar spindles remains unchanged, most are asymmetrical and abnormally elongated (Figure 5B, b, e, and h), often composed of large microtubule bundles with unfocused poles (Figure 5B, b). Interestingly, in contrast to control promet-

aphases, Dgrip84-depleted mitotic cells lack astral microtubules (Figure 5B, a–c, insets). Immunofluorescence analysis confirmed that Dgrip84 protein is substantially depleted from the mitotic poles and along spindle microtubules (Supplemental Figure B and Table 3). The two other components of the γ -TuSCs (γ -tubulin and Dgrip91) also fail to be recruited to the poles in $\sim 95\%$ of prometaphase figures (Supplemental Figure B and Table 3). Moreover, γ -tubulin staining is no longer detectable along spindle microtubules and to the midbody (our unpublished data). Therefore, the phenotypes resulting from the depletion of Dgrip84 in cultured S2 cells are in all respects similar to the phenotypes observed in *Dgrip84* mutants.

Because Dgrip84 depletion affects microtubule organization rather than their formation, we have next determined whether the poles retain some capacity to assemble microtubules. RNAi-treated cells still show a polar localization of Cnn (Figure 5B, b and c, and Table 3), but its recruitment is abnormal. Most cells containing bipolar spindles show two Cnn-labeled poles but with an asymmetrical distribution. When only one pole is stained, Cnn could occur as a single dot (Figure 5B, b), but in most cases three to four dots are observed (Figure 5B, c). The distribution pattern of Cnn might suggest an abnormal number of centriolar structures at the pole(s). Immunostaining for the Asp protein in Dgrip84-depleted cells gives strong support for this interpretation. This microtubule minus end marker is still clustered around the pole(s) (Table 3) but mostly occurs as one or multiple irregular structures (Figure 5B, e and f). This suggests that most of the microtubules retain their normal orientation but are organized from several microtubule nucleation centers. Electron microscopy (EM) analysis supports

Figure 5. Characterization of microtubule arrays after Dgrip84 depletion in cultured cells. (A) Dgrip84 level is strongly reduced after RNAi treatment. A total protein extract (50 μ g) of control cells (C) or of cells subjected to RNAi treatment (T) was analyzed by immunoblotting using affinity-purified Dgrip84 antibody and antibody directed against actin (internal loading control). (B and C) Dgrip84 is essential for the correct bipolar organization of the spindle. (B) Immunofluorescence analyses. The staining in green corresponds to the mitotic polar markers, Cnn (a–c) and Asp (d–f), or to the centromeric marker Cid (g–i). For a–c, white and black insets represent microtubule staining at the poles. For Cid staining, calcium pretreatment was performed to selectively disassemble astral microtubules. Bars, 5 μ m; For e, f, and h, same bar than in i. (C) Electron microscopy characterization of a monopolar spindle. Microtubules emanate from the centriolar microtubule triplets (arrows) at a polar region defined by a cluster of supernumerary centrioles. In this section, one microtubule (arrowheads) extends all the way from the centrioles to the kinetochore surface (Kt; surrounded in black) of the chromosome (Ch). Bar, 0.2 μ m. (D) Dgrip84 is required for the organization and the maintenance of the dynamics of interphase microtubules. Interphase microtubule array (α -tubulin immunostaining) is monitored after cold microtubule depolymerization (2 h at 4°C) followed by 2-min incubation at 22°C in control (a) or in Dgrip84-depleted cells (b). Bars, 5 μ m.

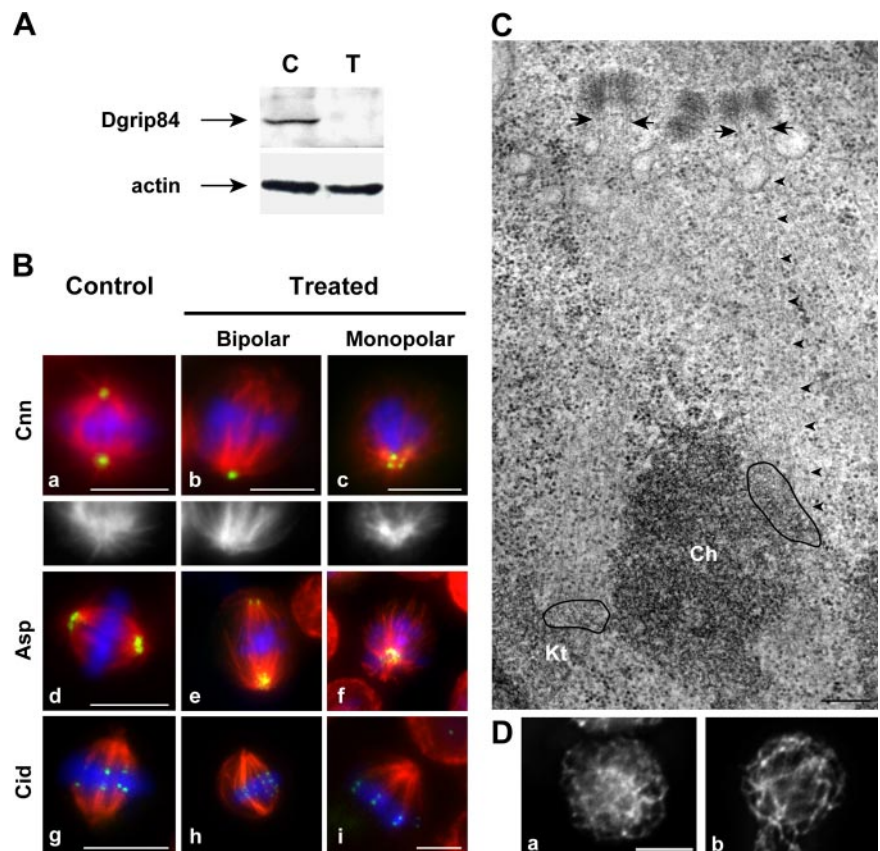


Table 3. Accumulation of centrosomal components in mitotic cells after depletion of Dgrip84 in cultured cells

Staining			Treated, %		
	Control, %		Monopolar	Bipolar	
	One pole	Both poles		One pole	Both poles
Dgrip84	4 (1–7)	88 (82–94)	5(1–9)	4 (1–7)	6 (2–10)
γ-Tubulin	3 (0–6)	97(94–100)	0	0	10 (3–17)
Dgrip91	7(2–12)	93 (88–98)	1(0–3)	2 (0–4)	0
Asp		100	100	1 (0–2)	98(96–100)
Cnn	5 (1–9)	95 (91–99)	100	35 (27–43)	65 (57–73)

Microtubules were stained with antibodies against α -tubulin and chromosomes with DAPI, whereas the spindle poles were immunostained with antibodies against Dgrip84, γ -tubulin, Dgrip91, Asp, or Cnn. $n > 10$.

the view that at least some poles possess supernumerary centrioles (Figure 5C). Interestingly, we could not clearly detect microtubules radiating from pericentriolar material, but numerous microtubules occur in continuity with the centriolar microtubule triplets (Figure 5C, arrows). Therefore, these abnormal centrosome-like structures retain some microtubule nucleation/organization capacity, but most likely through an atypical assembly pathway.

Because *Dgrip84* mutants show defects in prometaphase chromosome congression, we have next determined whether spindle microtubules in *Dgrip84*-depleted cells are able to establish stable end-on interactions with kinetochores using Cid as a centromere marker (Henikoff *et al.*, 2000). Before immunostaining, cells were preincubated with buffer containing calcium, which selectively dissociates nonkinetochore microtubules (compare Figure 5B, a with g). In cells showing either mono- or bipolar spindles, most kinetochores occur in the vicinity of microtubule bundles (Figure 5B, h–i), suggesting that kinetochores specifically associate with the plus ends of microtubule bundles. However, in contrast to control cells, in *Dgrip84*-depleted conditions, chromosomes are often found not aligned, closely positioned to the poles or distributed along the spindle. Similar results have been obtained after immunolabeling with the BubR1 marker (Supplemental Figure A). EM analysis further supports this result. In cells depleted for *Dgrip84*, bundles of microtubules running from the spindle pole made contact with individual chromosomes at the kinetochores (Figure 5C). Within these kinetochore-fibers, some microtubules seem to be elongated from centriolar triplets (Figure 5C, arrowheads). Even though the spindles are not fully functional, our results show that without detectable γ -TuSC components at the poles, the mitotic microtubules are still able to establish an interaction with the kinetochores. However, we cannot determine whether these microtubules are elongated at kinetochores (Maiato *et al.*, 2004), whether kinetochores capture microtubules abnormally assembled at centrosomal level, or whether both mechanisms contribute to kinetochore fiber assembly.

Dgrip84–RNAi Treatment Induces Subtle Effects on Interphase Microtubule Network

Depletion of *Dgrip84* does not induce obvious cytoskeletal defects during interphase. Microtubule arrays do not radiate from a unique site but is organized into a dense network emerging from multiple points. EM analysis shows the presence of centrosome-like structure, from which emerge or converge none or very few microtubules (our unpublished

data). All the pericentriolar markers we tested (γ -tubulin, *Dgrip84*, *Dgrip91*, *Dgrip75*, *Dgrip128*, and *Dgrip163*) do not stain this structure (our unpublished data), suggesting a centrosome with little or none pericentriolar material. The antibody against γ 37CD-tubulin, which exhibits biochemical characteristics of a centriolar component, decorates a spot at the periphery of the nuclear envelope only in late G₂ (Raynaud-Messina *et al.*, 2004). Together, our results suggest that interphase centrosomes are not fully competent for microtubule organization in S2 cells. The same hypothesis has been drawn for interphase centrosomes in larval brain cells (Martinez-Campos *et al.*, 2004). The existence of various focalization points and the complexity of interphase cytoskeleton organization could prevent the observation of a particular phenotype after inhibition of *Dgrip84*. After cold disassembly, microtubule regrowth occurred from several points without any preferential focalization, both in control and RNAi-treated cells (Figure 5D, a and b). However, a decrease in the number of regrowth points and the appearance of abnormally long cytoplasmic microtubules are noticed in *Dgrip84*-depleted cells (Figure 5D, b) compared with control. This phenotype occurs from 1 min at 22°C and maintained for longer regrowth times. These experiments point to a potential role of *Dgrip84* in the organization and the dynamics of the microtubules during interphase.

Dgrip84 Is Also Required for Spermatogenesis

Besides its role during mitosis, *Dgrip84* also seemed to be required for spermatogenesis because the adult *PG36* mutant males are sterile. Moreover, it is known that the spindle integrity checkpoint is less stringent during meiotic divisions than during neuroblast mitosis (Giansanti *et al.*, 2001). Therefore, this peculiarity has allowed us to gain some information on the evolution of mitotic figures in *Dgrip84*-silencing conditions. In wild-type, meiosis results in the formation of a cyst containing 64 spermatids. During the “onion stage,” each one consists of a single round phase-light haploid (N) nucleus associated with a phase-dense spherical mitochondrial aggregate of similar size, called the Nebenkern (Figure 6A, a). In adult mutant males, most of the scored cysts display only 16 spermatids, suggesting that *Dgrip84* is necessary for the proper completion of the two meiotic divisions. Moreover, almost all the spermatids show abnormalities in the number and/or size of Nebenkerns and nuclei. The most frequent abnormalities we noticed were 1) spermatids exhibiting a 1:1 ratio of nuclei to Nebenkern and nuclei with a size compatible with a tetraploid content (Figure 6A, b); 2) spermatids showing many nuclei of the same

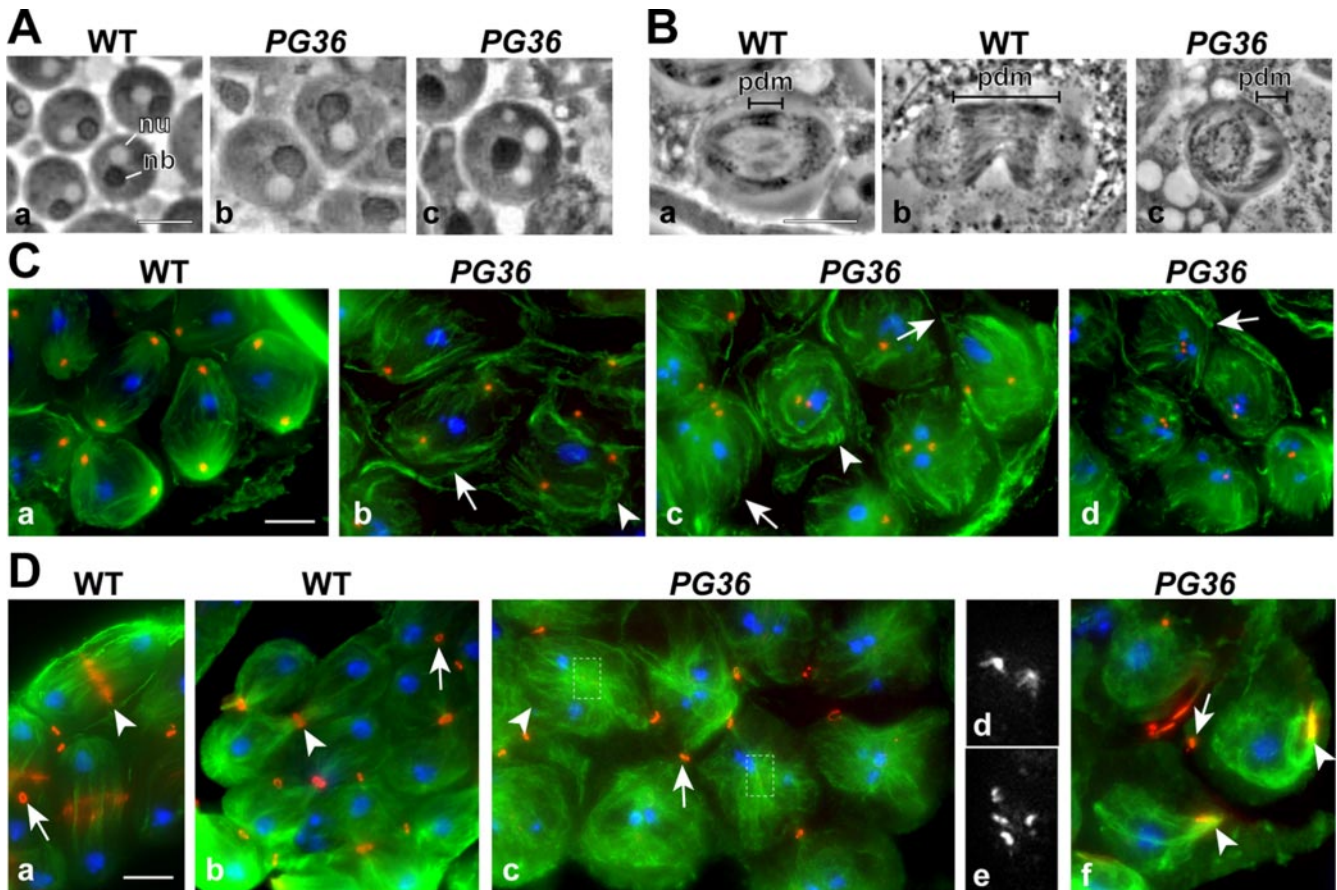


Figure 6. Meiotic phenotypes in *PG36* mutants. (A and B) Onion stage spermatids (A, a–c) and spindle organization (B, a–c) are abnormal as viewed by phase contrast microscopy. (A, a) Wild type spermatids (nu, nucleus; nb, Nebenkern). (A, a and b) *PG36* spermatids show abnormal phenotypes. (B, a and b) The dark band (pdm, phase dense material) outlining the equatorial region of the spindle corresponds to a system of parafusorial membranes and mitochondria that lines up along the nuclear membranes and microtubules. (B, a) Wild-type metaphase. (B, b) Wild-type telophase. (B, c) Conical-shaped structure observed in mutant meiosis. Cnn is shown in red and microtubules in green. a, Wild-type bipolar spindles. b and c, arrowheads, Mutant disorganized figures. c, arrows, Mutant conical-shaped structures. d, arrow, Mutant umbrella-shaped structures. (D) Pav-KLP recruitment is abnormal. Pav-KLP (red) recruitment in the ring canals is shown by arrows (a–c, and f). Pav-KLP is recruited on the central spindle in wild-type anaphases and telophases (a and b, arrowheads). In mutant cells (c–f), Pav-KLP is not detectable at the putative plus ends of microtubules in the umbrella-shaped structures (c, arrowhead), but it is recruited at the apex of the cones (f, arrowheads). d and e, To better visualize centrosomal Pav-KLP signal, the top- (d) and bottom (e)-selected regions of c have been 2.25-fold enlarged and signal enhanced. Bars, 10 μ m.

size or of various sizes but only one large Nebenkern (Figure 6A, c); and 3) spermatids with many Nebenkerns and one or several nuclei. According to previous studies (Sunkel and Glover, 1988), these abnormalities could be the consequences of defects in mitochondria localization on the spindle, failure of cytokinesis, or defects in the fidelity of chromosome segregation and karyokinesis.

To further investigate the organization of the microtubule cytoskeleton in mutant meiosis, observations of live spermatocytes by phase contrast microscopy and immunostaining of fixed preparations have been carried out. In wild-type primary spermatocytes, two asters are organized from the duplicated centrosomes and begin to separate at the onset of meiosis. In contrast, in mutant primary spermatocytes these two microtubule asters are not clearly detectable. Very few mutant meiotic cells seem to acquire a bipolar spindle organization (Figure 6C, b, arrow). Most are highly abnormal. Some are devoid of any polarity (Figure 6C, b and c, arrowheads); others exhibit monopolar-like structures, where microtubules seemed to be organized mainly from one or two

close asters (Figure 6C, d, arrow, and D, c, white rectangles). Among monopolar-like figures, some exhibit microtubules that diverge in an umbrella shape (Figure 6C, d, arrow), whereas in others, microtubules converge to the apex of a cone (Figure 6B, c, and C, c, arrows).

In wild-type primary spermatocytes, the two centrioles of each centrosome are always decorated by antibodies against Cnn (our unpublished data) (Li *et al.*, 1998), even though at this stage, only a small amount of pericentriolar material is present and closely associated to these structures (Martinez-Campos *et al.*, 2004). As cells enter meiosis, Cnn is massively recruited to the centrosome at the spindle poles of wild-type cells (Figure 6C, a). In mutant primary spermatocytes, Cnn staining is faint but often occurs as more than four dots per cell (our unpublished data), suggesting defects in centrosome segregation during the premeiotic mitosis. During meiosis, Cnn antibodies stain zero to six dots per cell, although two (Figure 6C, b–d) or four dots are generally observed. In cells with a disorganized microtubule cytoskeleton, these Cnn-containing bodies remained together or are

dispersed, whereas in most of the monopolar-like structures, they are located together at the asters. Moreover, the intensity of Cnn signals decreases in mutant meiotic cells. This observation is consistent with the weak labeling of the centrioles in mutant primary spermatocytes and suggests that Cnn recruitment does not occur properly. To understand whether mutant cells containing these monopolar-like microtubule structures could evolve toward cytokinesis, we have analyzed by immunostaining the recruitment of Pavarotti-KLP (Pav-KLP), a kinesin-like protein related to the mammalian MKLP-1, that is necessary for the completion of cytokinesis (Adams *et al.*, 1998) (Figure 6D). In wild-type male meiosis, Pav-KLP localizes to the ring canals, remnants of the contractile rings from earlier divisions, which occur as small red rings or dots (Figure 6D, a and b, arrows). It weakly labels the centrioles at the spindle poles. This protein also localizes to the central spindle in anaphase (Figure 6D, a, arrowhead) and concentrates in the midzone in telophase (Figure 6D, b, arrowhead). In mutant cells, the localization of Pav-KLP in the ring canals does not seem to be affected (Figure 6D, c and f, arrows), and its recruitment at the centrosomes still occurs (Figure 6D, d and e). However, Pav-KLP dots often localize within the same aster (Figure 6D, d and e) and an abnormal number of dots per cell can be detected (our unpublished data). These results support the hypothesis that Dgrip84 is necessary for proper centrosome separation. In the umbrella-shaped microtubule structures Pav-KLP is never detected at the putative plus end of microtubules (Figure 6D, c, arrowhead), whereas in cones Pav-KLP is associated with microtubules at the apex but never in a ring shape (Figure 6D, f, arrowheads). These observations suggest that at least some of the abnormal meiotic spindles in *Dgrip84* mutant cells are able to recruit a cytokinesis marker but in a highly inappropriate way.

DISCUSSION

The function of the three constituents of the γ -TuSC has been examined using genetic approaches only in unicellular organisms. These analyses must be further investigated in animals where γ -tubulin is assumed to be recruited to the centrosome as γ -TuRC. Functional analysis of γ -tubulin-associated proteins in metazoans has been mainly restricted to *Drosophila*: Dgrip91, a γ -TuSC protein, and Dgrip75, a protein that specifically belongs to the large complex. The study of Dgrip84, the third component of the small complex in *D. melanogaster*, completes the functional characterization of the γ -TuSC, allowing for the first time a comparison of the phenotypes resulting from the disruption of each γ -TuSC component in a same metazoan.

Their depletion has no obvious effect on the cytoskeleton organization during interphase. However, in Dgrip84- or γ -tubulin-depleted cells, regrowth experiments allow the appearance of abnormally long and less numerous cytoplasmic microtubules compared with control (this study; our unpublished data). It could be indicative of a role of γ -TuSC proteins in the assembly and the maintenance of the length of interphase microtubules. Long microtubules could also result from an increase of the concentration of free tubulin in consequence of low microtubule assembly. Similar phenotypes displaying long microtubules have been observed after mutations in the γ -tubulin encoding gene both in budding yeast and *A. nidulans* and in γ -TuSC components in *S. pombe* (Marschall *et al.*, 1996; Spang *et al.*, 1996; Vardy and Toda, 2000; Jung *et al.*, 2001). In S2 cells, this effect could be hidden because of the density and the complexity of microtubule arrays. Whatever the outset of interphase microtu-

bules in *Drosophila* cells, functional γ -TuSC seems required in some aspects of microtubule dynamics and organization.

Disruption of each γ -TuSC component promotes a moderate increase of the mitotic index (Barbosa *et al.*, 2000; Raynaud-Messina *et al.*, 2004; this study). The arrest is weak compared with the one induced after a microtubule poison treatment. Moreover, Dgrip84-RNAi depletion previously to drug exposure reduces significantly the blockage extent, suggesting a deregulation of the spindle checkpoint or an activation of another cell cycle checkpoint. Inactivation of *Drosophila* γ -tubulin, Dgrip84, or Dgrip91 results in large polyploid nuclei (Sunkel *et al.*, 1995; Barbosa *et al.*, 2000; this study). This observation strengthens the idea that when the γ -TuSC integrity is affected, cells could escape prematurely from the mitotic checkpoint. These results are consistent with several sets of data. Human γ -tubulin mutants expressed in *S. pombe* allow cytokinesis to proceed in spite of spindle abnormalities (Hendrickson *et al.*, 2001). Some alleles of the *A. nidulans* γ -tubulin gene exhibit a slight delay in mitosis and could enter interphase without correct division, even after a microtubule-destabilizing treatment (Prigozhina *et al.*, 2004). In *S. pombe*, mutations in genes encoding γ -tubulin interacting proteins bypass the spindle assembly checkpoint and cause the untimely activation of the septation initiation network (Vardy and Toda, 2000; Vardy *et al.*, 2002). Together, these results suggest a role of the γ -TuSC in the mitotic checkpoint control.

Mitotic figures of Dgrip84-depleted cells exhibit monopolar morphology or microtubule arrays that fail to define orientated polar structures and correct chromosome congression. This is also a characteristic feature of cells lacking Dgrip91 or γ -tubulin (Sunkel *et al.*, 1995; Barbosa *et al.*, 2000; Raynaud-Messina *et al.*, 2004). In monopolar spindles, Cnn- and Asp-labeling patterns are consistent with the presence of supernumerary centrioles at the poles. Electron microscopy analysis supports this view, suggesting that centrosomes fail to segregate. This abnormality has also been observed both in *Dgrip91* mutant neuroblasts and after 23C γ -tubulin depletion in S2 cells (Barbosa *et al.*, 2000; Raynaud-Messina *et al.*, 2004). Moreover, *Dgrip84* mutant spermatocytes exhibit monopolar structures. Similar figures, described in γ -tub23C and *Dgrip91* mutant spermatocytes (Sampaio *et al.*, 2001; Barbosa *et al.*, 2003), have been shown to be the consequence of the collapse of the two poles. *Drosophila* γ -TuSC assembly seems necessary for the proper separation of the centrosomes. Similar observations were obtained after γ -tubulin depletion in *Caenorhabditis elegans*, where separated asters are reapproaching in late prophase (Strome *et al.*, 2001). The simplest interpretation is that the microtubule network involved in the maintenance of centrosome separation is defective either in its dynamics or its density.

Our studies emphasize that, after Dgrip84 depletion, some assembly and organization of spindles still occur and point to mechanisms of nucleation independent of γ -tubulin complexes. Microtubule organization still takes place at the poles. However, instead of emerging from pericentriolar material, some microtubules occur in continuity with centriolar triplets. Although this mechanism is unlikely to be dominant in the wild-type spindle assembly, it could account for the nucleation of some microtubules in Dgrip84-depleted cells that are characterized by anastral spindles. Some microtubules are able to establish contact with kinetochores showing that they retain some of their functional properties. Strong BubR1 signal in RNAi-treated cells is consistent with kinetochore-microtubule occupancy but alterations in microtubule tension (Logarinho *et al.*, 2004).

Depletion of Dgrip84 prevents both γ -tubulin and Dgrip91 localization at the poles. Moreover, γ -tubulin depletion impairs Dgrip84 and Dgrip91 mitotic recruitment (Raynaud-Messina *et al.*, 2004; our unpublished data). Therefore, it is likely that γ -TuSC components must be assembled in complexes before relocalization to the poles or that γ -TuSC proteins are required for γ -tubulin polar attachment. This view, supported by studies in *S. cerevisiae* (Nguyen *et al.*, 1998), has been recently extended to mammalian cells: the γ -TuRCs could be anchored to the centrosome via interactions of GCP2 (Dgrip84 orthologue) and GCP3 (Dgrip91 orthologue) with the centrosomal proteins CG-NAP and pericentrin (Takahashi *et al.*, 2002; Zimmerman *et al.*, 2004). In *Drosophila*, the calmodulin-binding protein CP309 has been proposed to tether the γ -TuRC to the centrosome through direct binding with γ -TuSC components (Kawaguchi and Zheng, 2004). So, the γ -TuSC grip-motif proteins seem essential, due to their critical role in the attachment of γ -tubulin complexes to the pericentriolar matrix. In contrast, the proteins specific for the γ -tubulin large complexes characterized so far, like *Drosophila* Dgrip75 and *S. pombe* Alp16p (Dgrip163 orthologue) or Gfh1p (Dgrip75 orthologue), are not essential for cell viability (Fujita *et al.*, 2002; Venkatram *et al.*, 2004). It could be hypothesized that these γ -tubulin partners play a role in the organization or in the dynamics of a subset of microtubules.

Analysis of meiosis in mutant spermatocytes reveals that Dgrip84, like Dgrip91 and γ -tubulin (Sampaio *et al.*, 2001; Barbosa *et al.*, 2003), is required for completion of the two meiotic divisions. In the absence of one of the γ -TuSC components, abnormal mitotic spindles can evolve toward a central spindle-like structure as viewed by the recruitment of different markers (Polo Kinase, Klp3A [Sampaio *et al.*, 2001], Asp [Barbosa *et al.*, 2003], Pav-KLP [Barbosa *et al.*, 2003]; this study). Nevertheless, these proteins exhibit abnormal localization and cytokinesis is strongly asymmetrical and/or clearly abortive.

In conclusion, we present the characterization of the third and last component of a metazoan γ -TuSC. Together with previous studies, our data strongly suggest that the integrity of this complex must be maintained to ensure γ -tubulin recruitment, proper microtubule organization and an efficient mitotic checkpoint. In contrast, the depletion of γ -TuRC-specific proteins does not impair viability and γ -tubulin accumulation. We suggest that the γ -TuSC could be a universal and minimal subunit required for γ -tubulin recruitment to the cytoskeleton structures and proper microtubule nucleation in eukaryotic cells.

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